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Induction of Apoptosis in Cord Blood Lymphocytes by HHV-6

Ryoji Ichimi, Toshimi Jin-no, and Masahiro Ito*
Department of Pediatrics, Mie University School of Medicine, Mie, Japan

Apoptosis induced by human herpesvirus 6 (HHV-6) in cord blood lymphocytes was investigated. Cord blood mononuclear cells (CBMC) prestimulated with phytohemagglutinin (PHA) were infected with HHV-6 and cultured with interleukin 2 (IL-2) for 5 days. Apoptosis was investigated by cell cycle analysis, terminal deoxytransferase (TdT)-mediated dUTP nick end-labeling (TUNEL) assay, and staining with monoclonal antibody APO2.7 reacting with 7A6 antigen. The percentage of the hypodiploid fraction by cell cycle analysis and the percentage of apoptosis determined by TUNEL assay were significantly higher in HHV-6–infected CBMC compared with uninfected CBMC. 7A6 antigen, induced on the mitochondria membrane in apoptotic cells, were mainly expressed in CD4+ cells. 7A6 antigen was also detected in HHV-6–infected cells determined by monoclonal antibody OHV-3 reacting with HHV-6 glycoprotein. These data indicated that HHV-6 induced apoptosis in HHV-6–infected cells after stimulation with IL-2 for 5 days. The addition of anti-Fas antibody, anti-Fas ligand antibody, and anti-TNF-α antibody did not affect the induction of apoptosis by HHV-6, indicating that the Fas-Fas ligand pathway and TNF pathway did not contribute to the apoptosis induced by HHV-6. ***J. Med. Virol. 58:63–68, 1999.***

KEY WORDS: apoptosis; cord blood; HHV-6

INTRODUCTION

Human herpesvirus 6 (HHV-6) is known to be a causative agent of exanthema subitum [Yamanishi et al., 1988], a common febrile illness with a rash in infancy. HHV-6 has also been associated with pneumonitis [Carrigan et al., 1991], hepatitis [Dubedat S et al., 1989], mononucleosis-like illness [Steeper et al., 1990], and fatal hemophagocytic syndrome [Huang et al., 1990]. HHV-6 remains latent after primary infection and reactivates in an immunocompromised state, as do other human herpesviruses. HHV-6 has been associated with bone marrow suppression after bone marrow transplantation [Carrigan et al., 1994]. It has been observed that HHV-6 can infect various cell types, although this virus mainly infects and replicates in CD4+ lymphocytes [Frenkel et al., 1990] and has an immunosuppressive effect on T-cell functions, including IL-2 synthesis and cell proliferation [Flamand et al., 1995].

Human herpesviruses, such as herpes simplex virus (HSV)-1, varicella-zoster virus, and Epstein-Barr virus, induce apoptosis [Kawanishi, 1993; Sadzot-Delvaux et al., 1995; Ito et al., 1997a, 1997b]. Induction of apoptosis can limit virus production and reduce or eliminate the spread of virus in the host [Teodoro et al., 1997]. Influenza virus induces apoptosis in bronchial epithelial cells and alveolar cells [Mori et al., 1995], and measles virus induces apoptosis in thymocytes [Auwaerter et al., 1996]. These data indicate that apoptosis may contribute to the pathogenesis induced by infection. It has been reported that HHV-6 can induce apoptosis of the human T-cell line, indicating that cell death of CD4+ lymphocytes mediated by HHV-6 is due to apoptosis [Inoue et al., 1997]. However, the effect of HHV-6 infection on lymphocytes from neonates has not been fully investigated. To clarify the effect of HHV-6 on the immune system, it is important to study the biological effect of HHV-6 on human lymphocytes. In this study, the induction of apoptosis by HHV-6 in T-lymphocytes from neonates was investigated.

MATERIALS AND METHODS

Preparation of Cord Blood Lymphocytes

Cord blood was obtained from the placental end of the cord at full-term birth and cord blood mononuclear cells (CBMC) were separated by Ficoll-Hypaque (Histopaque 1077, Sigma, St. Louis, MO) gradient centrifugation. CBMC at the interface were collected and washed three times with RPMI-1640 (Gibco-BRL, Grand Island, NY) and suspended in RPMI-1640 with 10% heat-inactivated fetal bovine serum (FBS) (Gibco).

*Correspondence to: Dr. Masahiro Ito, Department of Pediatrics, Mie University School of Medicine, 2-174 Edobashi, Tsu, 514-8507 Mie, Japan. E-mail: virus@clin.medic.mie-u.ac.jp

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Preparation of HHV-6 Virus Stock

The Hashimoto strain of HHV-6 variant B was inoculated onto CBMC, which were cultured with 5-μg/ml phytohemagglutinin (PHA) (Sigma) in RPMI-1640 (Gibco) with 10% FBS (Gibco) for 3 days. Then, HHV-6–infected CBMC were cultured with 10 U/ml of human recombinant interleukin-2 (IL-2) (Genzyme, Cambridge, MA). When the cytopathic effects were maximal, CBMC were centrifuged at 3,000 rpm for 15 min and the supernatants were used for HHV-6 virus stock. Virus stock was frozen at −80°C until use. Uninfected CBMC were similarly cultured and the supernatant of uninfected cells was used for mock-infection.

Inactivation of Virus

Virus stock was placed in a glass petri dish and exposed to UV light or was heat-inactivated by exposure to 60°C for 1 hr.

Preparation of CD4- and CD8-Rich Lymphocytes

CD4- and CD8-rich lymphocytes were obtained using DYNABEADS M-450 CD4 and M-450 CD8 (Dynal, Skoyøn, Norway). Briefly, 1×10^7 of CBMC and 75 μl of beads M-450 CD4 or M-450 CD8 were mixed (bead-to-cell ratio of 3:1) on ice for 30 min. Rosette cells with beads were collected with a magnetic particle concentrator (MPC) and detached by mixing 10 μl of DETACHaBEAD for 1 hr. Cells collected by removing the detached beads using the MPC were used for the CD4- and CD8-rich populations. These procedures resulted in >95% CD4+ cells and CD8+ cells, respectively.

Stimulation of Lymphocytes Before HHV-6 Infection

CBMC, CD4+ lymphocytes, or CD8+ lymphocytes suspended in RPMI-1640 with 10% FBS were cultured with 5-μg/ml PHA for 3 days. After culture, viable cells were separated by Ficoll-Hypaque (Histopaque 1077, Sigma) gradient centrifugation. Viable cells were resuspended in RPMI-1640 with 10% FBS at a concentration of 5×10^6/ml.

HHV-6 Infection

Lymphocytes (5×10^6) were centrifuged at 1,500 rpm for 5 min. The pelleted cells were resuspended in 0.5-ml HHV-6 stock or with control supernatant, then adsorbed for 90 min at 37°C with occasional mixing. The cells were washed three times with RPMI-1640 to remove unabsorbed virus and resuspended at a concentration of 1×10^6/ml in RPMI-1640 supplemented with 10% FBS.

Stimulation of Lymphocytes After HHV-6 Infection

HHV-6–infected or uninfected cells were cultured at 37°C in 5% CO_2 in the presence or absence of human recombinant interleukin-2 (IL-2) (Genzyme)or 5-μg/ml PHA for 5 days.

Cell Cycle Analysis

Cells within the hypodiploid peak show a DNA ladder typical of apoptosis [Nicoletti et al., 1991]. Cultured lymphocytes (1×10^6 cells) were washed with phosphate-buffered saline (PBS) and resuspended in 75% ice-cold ethanol and kept at −20°C for 30 min, then washed with PBS. The cells were incubated with 100-μg/ml ribonuclease A (Sigma) at 37°C for 30 min. The cells were resuspended in 0.5 ml of staining solution (0.1-mg/ml propidium iodine in PBS; Sigma) in the dark at room temperature for 30 min and then analyzed with a FACScan (Becton-Dickinson) using the CeLLFIT program (Becton-Dickinson). In the fluorescence histogram, the percentage of cells in S-phase or with a hypodiploid peak (apoptotic cell nuclei) was calculated using LYSIS software (Becton-Dickinson).

Detection of Apoptosis by TdT-Mediated dUTP Nick End-Labeling (TUNEL) Assay

Labeling of DNA strand breaks with fluorescein and analysis by flow cytometry allows for quantitative analysis of apoptosis [Gavrieli et al., 1992]. DNA ends (3′-OH) generated by DNA fragmentation were nick end-labeled with fluorescein isothiocynate (FITC)-conjugated dUTP that was introduced by terminal deoxytransferase (TdT) using an in situ cell death detection kit (no. 1684795, Boehringer Mannheim, Germany). The cells were fixed in 4% paraformaldehyde in PBS at room temperature for 30 min, washed in PBS, and permeability was enhanced by treatment with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice. Cells were then washed twice in PBS and resuspended in the TUNEL reaction mixture (TdT, FITC-conjugated dUTP) and incubated for 60 min at 37°C in a humidified atmosphere in the dark. Cells were washed twice with PBS and analyzed by flow cytometry using the CELLQuest computer software program (Becton Dickinson).

Staining of HHV-6 Antigen With Monoclonal Antibody OHV-3

Cells were washed with PBS and resuspended in 100 μl of PBS. Next, 10 μl of mouse monoclonal antibody (MoAb) to HHV-6 variant B (OHV 3, provided by Dr. K. Yamanishi, Osaka University) or control mouse monoclonal IgG1 antibody (DAKO) was added. The cells were washed three times with PBS and incubated with fluorescein isothiocyanate (FITC)-conjugated affinity purified $F(ab')_2$ goat antimouse IgG (Dako Japan, Kyoto, Japan) for 1 hr at 37°C. The cells were again washed with PBS three times and analyzed by FACScan (Becton Dickinson) using the CELLQuest software program (Becton Dickinson).

Staining of 7A6 Antigen With Monoclonal Antibody AP02.7

MoAb APO2.7 reacts with a 38-kDa protein (termed 7A6 antigen) localized to the membrane of mitochon-

TABLE I. Induction of Apoptosis in Different Culture Conditions

Stimulation before infection	Stimulation after culture[b]	Percent net apoptosis[b] TUNEL assay	Percent net apoptosis[b] Cell cycle analysis
PHA[a]	IL-2 100U/ml	24.8	26.5
PHA	IL-2 50 U/ml	14.5	12.5
PHA	IL-2 10 U/ml	5.8	2.8
PHA[c]	PHA (5 μg/ml)	0	0
PHA	none	0	0
none	PHA (5 μg/ml)	0	0
none	IL-2 100U/ml	0	0
none	none	0	0

[a]HHV-6–infected CBMC or uninfected CBMC were cultured as indicated for 5 days. Apoptosis was analyzed by TUNEL assay and cell cycle analysis.
[b]% net apoptosis = % apoptosis in HHV-6–infected cells – % apoptosis in uninfected cells.
[c]CBMC were cultured with PHA (5 μg/ml) for 3 days.

dria in apoptotic cells [Zhang et al., 1996]. Cells were washed with PBS resuspended in 100 μl of PBS; 5 μl of phycoerythrin (PE)-conjugated MoAb APO2.7 (clone 2.7A6A3, mouse IgG1; Immunotech, Marseille, France) was added and incubated for 15 min at room temperature. The cells were then washed with PBS three times and analyzed by FACScan (Becton Dickinson) using the CELLQuest software program (Becton Dickinson). In some experiments, cells were stained with MoAb OHV3 after staining with MoAb APO2.7 and simultaneously stained with MoAb APO2.7 and FITC-labeled anti-CD3, or CD4 or CD8 MoAb (Becton Dickinson).

Blocking Experiments

Fas ligand (FasL) induced apoptosis in Fas-expressing cells [Yonehara et al., 1989; Suda et al., 1993]. To determine the role of Fas-FasL interaction, antihuman Fas mouse MoAb (clone ZB4, mouse IgG1, MBL) and antihuman FasL MoAb (clone 4A5, hamster IgG, MBL) were used. Anti-TNF-α monoclonal antibody (neutralizing anti-h, clone 195, mouse IgG3, Boehringer Mannheim) was also used to determine the role of TNF-α. These monoclonal antibodies have neutralizing activity and were added to the culture shortly after infection with HHV-6. Apoptosis in the presence or absence of these antibodies were analyzed by TUNEL assay and cell cycle analysis.

RESULTS

Induction of Apoptosis by HHV-6 Under Different Culture Conditions

When fresh CMBC were infected with HHV-6 and cultured with IL-2 or PHA for 5 days, no significant apoptosis was observed. When CBMC precultured with PHA for 3 days were infected with HHV-6 and cultured with PHA for 5 days, apoptosis was not observed. Apoptosis was induced when cells were infected with HHV-6 after prestimulation with PHA and cultured in the presence of IL-2 for 5 days. The magnitude of apoptosis was dependent on the dosage of IL-2 (Table I). Representative cell cycle analysis and TUNEL assay is shown in Figure 1. The percentages of the hypodipliod fraction in HHV-6–infected and uninfected CBMC cultured with IL-2 were 18.9% and 4.7%, respectively. The percentages of apoptotic cells in HHV-6–infected and uninfected CBMC cultured with IL-2 by TUNEL assay were 51.4% and 6.4%, respectively. Mean percentage of the hypodiploid fraction and the percentage of apoptosis from different experiments are shown in Figure 2. The percentage of the hypodiploid fraction and the percentage of apoptosis was significantly greater in HHV-

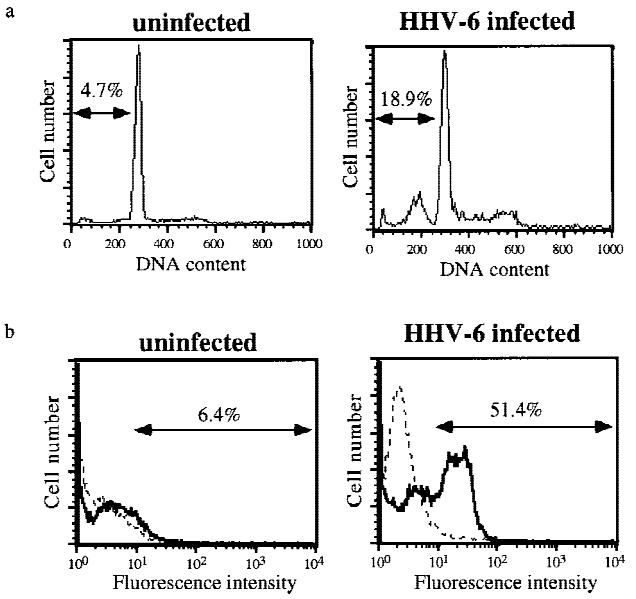

Fig. 1. Cord blood mononuclear cells (CBMC) were cultured with 5-μg/ml PHA for 3 days and infected with HHV-6. HHV-6–infected and uninfected CBMC were cultured with 100-U/ml IL-2 for 5 days. **A:** Cell cycle analysis. Data indicated the percentage of hypodiploid fraction. **B:** TUNEL assay. Data indicated the percentage of apoptosis. Cells were stained with FITC-conjugated dUTP by TdT (——) or without TdT (– –).

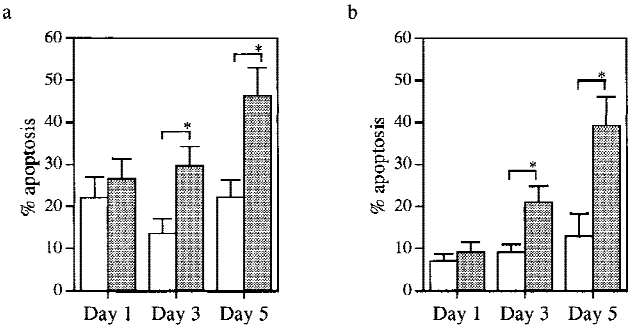

Fig. 2. CBMC were cultured with 5-μg/ml PHA for 3 days and infected with HHV-6. HHV-6–infected and uninfected CBMC were cultured with 100-U/ml IL-2 for 1, 3, or 5 days. Data indicated mean % apoptosis ± SE by TUNEL assay **(A)** and cell cycle analysis **(B)** from 10 different experiments. $P < 0.05$ compared with uninfected cells (Wilcoxon signed rank test). ▩: HHV-6–infected cells; □: uninfected cells.

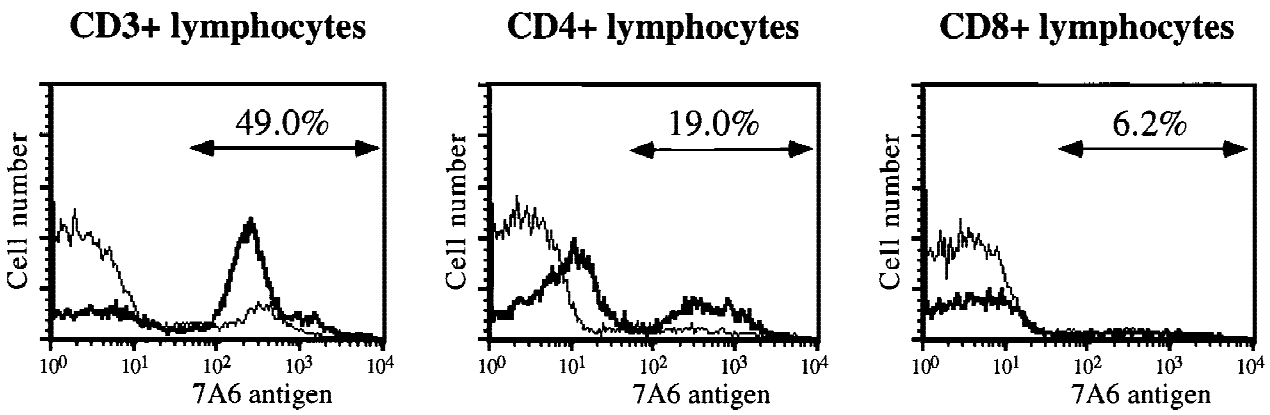

Fig. 3. Expression of 7A6 antigen in HHV-6–infected and uninfected CBMC. HHV-6–infected CBMC (▬) and uninfected CBMC (——) were stained with FITC-labeled anti-CD3, -CD4, or -CD8 monoclonal antibody and PE-labeled monoclonal antibody APO 2.7. $CD3^+$, $CD4^+$, and $CD8^+$ cells were gated and the expression of 7A6 antigen in the gated cells were indicated as a histogram. Data indicated the percentage of 7A6 antigen-positive cells in HHV-6–infected cells. Representative data were taken from three different experiments.

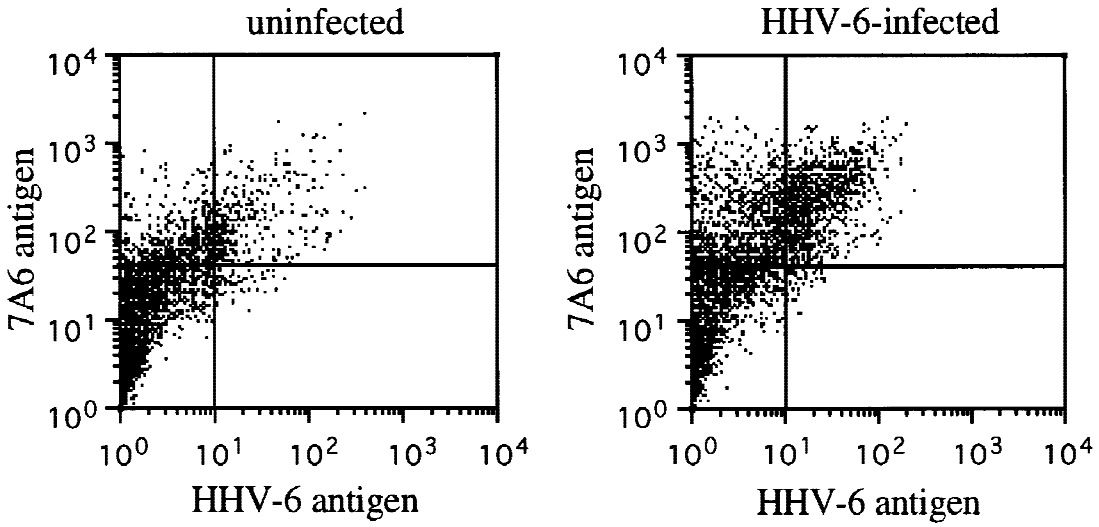

Fig. 4. Expression of HHV-6 antigen and 7A6 antigen in HHV-6–infected cells. HHV-6–infected CBMC **(right)** and uninfected CBMC **(left)** were stained with MoAb OHV-3 and FITC-labeled antimouse IgG, then with PE-labeled MoAb APO2.7 Representative data were taken from three different experiments.

6–infected cells than uninfected cells 3 and 5 days after infection.

Induction of Apoptosis in CD4+ Lymphocytes

HHV-6–infected and uninfected CBMC were simultaneously stained with anti-CD3, -CD4, or -CD8 antibody and APO2.7 antibody after 5 days' culture with IL-2. CD3-, CD4-, or CD8-positive cells were gated and the expression of 7A6 antigen in the gated cells was shown in a histogram (Fig. 3). 7A6 antigen was detected in CD3+ and CD4+ cells, but not in CD8+ cells. CD4+ and CD8+ lymphocytes were cultured with PHA (5 μg/ml) for 3 days. HHV-6–infected and uninfected CD4+ and CD8+ lymphocytes were cultured with IL-2 for 5 days. Percent net apoptosis (% apoptosis in HHV-6–infected cells −% apoptosis in uninfected cells) by TUNEL assay and cell cycle analysis in CD4+ cells was 27.9 ± 6.8 and 34.5 ± 5.2, respectively. Percent net apoptosis by TUNEL assay and cell cycle analysis in CD8+ cells was 2.5 ± 2.1 and 1.3 ± 0.5, respectively.

Expression of 7A6 Antigen in HHV-6 Antigen-Positive Cells

HHV-6–infected and uninfected CBMC were simultaneously stained with OHV-3 antibody and APO2.7 antibody after a 5-day culture with IL-2. Among HHV-6–infected cells, 20.3% expressed both HHV-6 antigen and 7A6 antigen and 15.9% expressed only 7A6 antigen. Among uninfected cells, 10.4% expressed 7A6 antigen (Fig. 4).

Effect of Anti-Fas, Fas-Ligand, and TNF-α Antibody on Apoptosis Induced by HHV-6

Apoptosis was induced in HHV-6–infected CBMC in the presence of anti-Fas antibody, anti-FasL antibody,

mixture of anti-Fas and anti-FasL antibody, and anti-TNF-α antibody (data not shown).

DISCUSSION

It was demonstrated that HHV-6 induced apoptosis in CD4 lymphocytes from cord blood when stimulated with IL-2. There is the possibility that soluble factors included in the HHV-6 virus stock may have induced the apoptosis, although this is unlikely because UV or heat-inactivated virus stock did not induce apoptosis (data not shown). Apoptosis occurred only when HHV-6–infected cells were cultured with IL-2, indicating that the activation signal induced by IL-2 is required for apoptosis. HHV-6 usually can be isolated from blood samples when stimulated with IL-2 or IL-2 plus PHA. HHV-6 did not replicate unless lymphocytes were activated.

Based on these results, it is possible to hypothesize that apoptosis is induced in HHV-6–infected cells. We investigated whether apoptosis occurred in both HHV-6–infected and uninfected cells. It has been reported that monoclonal antibody APO2.7 is reactive with a 38-kDa protein (7A6 antigen) localized to the membrane of mitochondria in apoptotic cells and 7A6 antigen is not on the normal cell surface or permeabilized cells [Zhang et al. 1996]. 7A6 antigen is detected when cells were just beginning to undergo apoptosis, suggesting that the expression of 7A6 Ag represents an early event in apoptosis. 7A6 Ag is also detected in nonpermeabilized cells at a late stage of apoptosis. Therefore, MoAb APO 2.7 was used to define expression of 7A6 antigen in HHV-6 antigen-positive cells. Monoclonal antibody OHV3 reacts with 98-K and 92-K glycoproteins of HHV-6 [Okuno et al., 1990]. It was found that apoptosis was induced in HHV-6 antigen positive cells. HHV-6 mainly infects and replicates in $CD4^+$ lymphocytes and apoptosis was observed in $CD4^+$ cell populations, suggesting that HHV-6 induced apoptosis in HHV-6–infected cells.

The mechanism by which viral infection induced apoptosis is still not known. One possibility is that $CD8^+$-lymphokine-activated killer cells (LAK) induced by IL-2 kill HHV-6–infected $CD4^+$ cells [Wang et al., 1998]. However, this is unlikely because $CD4^+$ lymphocytes underwent apoptosis in the absence of $CD8^+$ lymphocytes. Inoue et al. [1997] reported that apoptosis was observed in uninfected cells and TNF-α augmented apoptotic cell death in the $CD4^+$ human T-cell line by HHV-6 inoculation. They concluded that HHV-6 induces apoptosis in $CD4^+$ lymphocytes by an indirect mechanism. It has been reported that expression of Fas on cell surface and mRNA level was increased in influenza virus-infected cells [Wada et al., 1995]. In our study, pathways of Fas-Fas ligand and TNF do not contribute to the induction of apoptosis in HHV-6–infected cells. Recently, caspase-activated deoxyribonuclease (CAD) and its inhibitor (ICAD) was found [Enari et al., 1998]. However, the mechanism of the effect of viral infection on caspase family proteases is still unknown. Further study is needed to clarify this mechanism.

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